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In Vitro Effect of Tinidazole and Furazolidone on Metronidazole-Resistant *Trichomonas vaginalis*

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***Trichomonas vaginalis* is a common sexually transmitted protozoan parasite. Although often considered simply a nuisance infection, *T. vaginalis* has been implicated in premature rupture of placental membranes and increases in the risk of acquiring human immunodeficiency virus. Metronidazole, a 5-nitroimidazole, is currently the drug of choice to treat *T. vaginalis* infection. Because some patients have severe reactions to metronidazole and others are infected with metronidazole-resistant *T. vaginalis*, we were prompted to investigate alternative therapies. Tinidazole, another 5-nitroimidazole used in other countries to treat *T. vaginalis* infections, and furazolidone, a nitrofuran presently used to treat giardiasis and infections with some anaerobic enteric bacteria, were investigated for effectiveness against 9 metronidazole-susceptible and 12 metronidazole-resistant *T. vaginalis* patient isolates. The in vitro aerobic and anaerobic minimum lethal concentrations (MLC) and the time for drug efficacy were determined. Tinidazole killed the metronidazole-susceptible isolates at a low MLC but was effective against only 4 of the 12 metronidazole-resistant isolates. In contrast, furazolidone was effective at a low MLC for all isolates. When tinidazole was effective, it required >6 h to kill trichomonads. However, furazolidone killed both metronidazole-susceptible and resistant trichomonads within 2 to 3 h of exposure. These data suggest that furazolidone may be a good candidate for treating metronidazole-resistant trichomoniasis and that further investigation of this drug is warranted.**

An estimated 3 million American women (approximately 180 million women worldwide) are infected with *Trichomonas vaginalis* every year. It is the most common cause of vaginitis and is considered to be the most prevalent nonviral sexually transmitted disease agent (14). Infection with this organism has been linked to various additional pathologic manifestations, including cervical neoplasia (9, 19, 55), atypical pelvic inflammatory disease (2), and tubal infertility (10), and has been reported to be a risk factor in the development of post-hysterectomy cuff cellulitis (46). Infection with *T. vaginalis* has been linked to premature rupture of placental membranes, premature labor, and low birth weight (4, 33). *T. vaginalis* infection has also been reported to increase intrauterine transmission of cytomegalovirus (8) and elevate the risk of acquiring human immunodeficiency virus (22).

Metronidazole has been the drug of choice for treating trichomoniasis since 1959 and is currently the only drug licensed for this purpose in the United States. The recommended metronidazole regimen results in cure rates of approximately 95% (3). Yet clinical resistance to metronidazole therapy, defined as failure to cure the infection after at least two consecutive courses of metronidazole, was reported as early as 1962 (42). In most cases, metronidazole resistance has been considered the result of (i) insufficient absorption and/or transport of drug to the site of infection, (ii) the presence of bacterial flora that inactivate the drug (30), (iii) reinfection, or (iv) noncompliance with drug therapy. With increasing numbers of reports of clinically metronidazole-resistant trichomoniasis (6, 7, 11, 13, 15, 21, 27, 29, 32, 38, 41, 51) that have analyzed drug absorption and transport, that have investigated the effect of bacterial flora, and that have hospitalized patients to guard against reinfection and ensure compliance with therapy, clinical metronidazole-resistant trichomoniasis is now known to be primarily a parasite-dependent phenomenon.

In 1989, the Centers for Disease Control (CDC) approximated that 5% of all *T. vaginalis* patient isolates displayed some level of resistance to metronidazole (28). The prescribed regimen for patients with resistant infections (longer treatment with higher dosage of drug) is effective for only 80% of the patients with metronidazole-resistant trichomoniasis (27, 28). In addition to these patients with metronidazole-resistant *T. vaginalis* infections, other patients have adverse reactions to high doses or are allergic to metronidazole (50). On the basis of these percentages, it can be predicted that there are up to 30,000 U.S. trichomoniasis cases per year that cannot be treated with metronidazole. These numbers indicate a need for an alternative therapy for this infection.

MATERIALS AND METHODS

Organisms. A total of 21 *T. vaginalis* isolates were tested. Eight of the isolates analyzed were well characterized as metronidazole resistant (RU368, RU376, RU393, RU381, RU370, RU362, and CDC085) or metronidazole susceptible (CDC520) by Müller et al. (37). These isolates had been cryopreserved and stored at the CDC in liquid nitrogen. A second group of eight isolates (EMU1, EMU2, EMU15, EMU17, EMU29, EMU31, EMU71, and EMU74) were collected by Mark Martens (Emory University School of Medicine, Atlanta, Ga.) from patients seen at the Grady Memorial Hospital Gynecology Clinic in the fall of 1994. All eight of these isolates were susceptible to metronidazole treatment. A third group consisted of five specimens that were sent to the CDC for metronidazole susceptibility testing after being isolated from patients with clinical metronidazole-resistant trichomoniasis (CDC698, MSA800, MSA802, MSA803, and MSA804). The patients from which these organisms were isolated had gone through at least two consecutive metronidazole regimens without cure. The last two groups of organisms had been kept in liquid culture no longer than 2 months.

Organisms were maintained in Trypticase-yeast-maltose medium (TYM; pH 6.0) and 10% heat-inactivated fetal bovine serum at 37°C (5). Samples of each isolate were stored in TYM containing 0.1% agar, 10% heat inactivated fetal bovine serum, and 10% dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, Mo.) in liquid nitrogen (16).

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Metronidazole, tinidazole, and furazolidone (Sigma) were dissolved in 100% DMSO. Metronidazole and tinidazole went readily into solution in DMSO, but furazolidone was less soluble. To obtain better solubility of furazolidone, the DMSO solution was autoclaved at 121°C for 15 min at 15 lb/in^2. The drug-DMSO solution was then diluted in TYM (pH 6.0).

Trichomonads (10^4) were added to serial twofold dilutions (400 to 0.2 μg/ml) of the antimicrobial agents in 96-well U-bottom plates (catalog no. 3799; Costar Corp, Cambridge, Mass.). All experiments were run twice in triplicate with DMSO standard controls under both aerobic and anaerobic conditions. Anaerobic conditions were created in a GasPak Jar by using CO_2 generators (catalog no. B71040; Fisher Scientific, Pittsburgh, Pa.) which were monitored with anaerobic indicator strips (catalog no. B71051; Fisher Scientific). Plates were incubated at 37°C for 48 h and then examined microscopically with an inverted phase-contrast microscope. The lowest concentration of drug in which no motile organisms were observed is defined as the minimum lethal concentration (MLC) (32).

Because the environment in which *T. vaginalis* resides in the host is in a constant state of flux, it was important to determine (i) how quickly the drug affected the organism and (ii) if the removal of residual drug after various exposure times would have any effect on the organisms' viability. To do this, the 96-well plate assay described above was used; however, the plates were viewed at various times (3, 4, 5, 6, 9, 12, 18, 21, 24, 30, 36, 42, and 48 h) throughout the experiment to determine which concentration of drug at each particular time would be considered the MLC.

Every 6 h throughout the experiment, the organisms in the wells containing 1 dilution before (i.e., a drug concentration higher than) the MLC, the MLC, and the 4 dilutions after (i.e., drug concentrations lower than) the MLC were removed from the original plate, transferred to a new plate, and washed three times with TYM (pH 6.0). Fresh medium minus drug was added, and the organisms were incubated at 37°C in an anaerobic environment. After 48 h, the new plate was examined microscopically for the presence of viable organisms. The lowest drug concentration in which no motile organisms were observed was noted.

RESULTS

Clinical metronidazole resistance of *T. vaginalis* isolates is correlated to the 48-h in vitro aerobic MLC (28). Isolates from metronidazole-susceptible cases usually have aerobic MLCs of <50 μg/ml, isolates with MLCs of 50 μg/ml are correlated with very low levels of clinical resistance, and isolates from clinically resistant cases tend to have MLCs of ≥200 μg/ml (28). High 48-h anaerobic MLCs (>12.5 μg/ml) have also been correlated with clinical metronidazole resistance; however, this correlation is not as reliable as that with aerobic MLC values (28). Nonetheless, because *T. vaginalis* is a facultative anaerobe, the assay was also conducted in the absence of oxygen to evaluate the efficacy of these drugs under conditions more conducive to its growth. The 48-h aerobic and anaerobic MLCs of furazolidone, metronidazole, and tinidazole for all 21 isolates are shown in Fig. 1. Forty-eight-hour aerobic metronidazole MLCs (Fig. 1a) for isolates 1 to 12 (clinically metronidazole resistant) were 50 μg/ml or greater, while those for isolates 13 to 21 (metronidazole susceptible) were 25 μg/ml or less (Fig. 1a). Tinidazole showed high 48-h aerobic MLCs (≥50 μg/ml) for 9 of the 12 metronidazole-resistant isolates (organisms 1 to 7 and 10 and 12), while those for all other isolates were <50 μg/ml. In contrast, furazolidone was active against both the clinically metronidazole-susceptible organisms (isolates 13 to 21) and the metronidazole-resistant organisms (isolates 1 to 12), with 0.8 μg/ml being the highest concentration needed. The anaerobic MLCs of metronidazole and tinidazole were lower than their respective aerobic MLCs (Fig. 1b). These differences are due to the presence of oxygen interfering with the 5-nitroimidazole toxicity pathway (35). Metronidazole anaerobic MLCs for 9 of the 12 isolates defined as metronidazole resistant in the 48-h aerobic MLC assay (isolates 1 to 8 and 12) were higher than those for the organisms clinically defined as susceptible. Only four of the nine isolates with aerobic tinidazole MLCs of ≥50 μg/ml had high anaerobic tinidazole MLCs. Again, furazolidone effectively killed all isolates at low concentrations of drug. The highest concentration necessary in the anaerobic environment was 3.1 μg/ml.

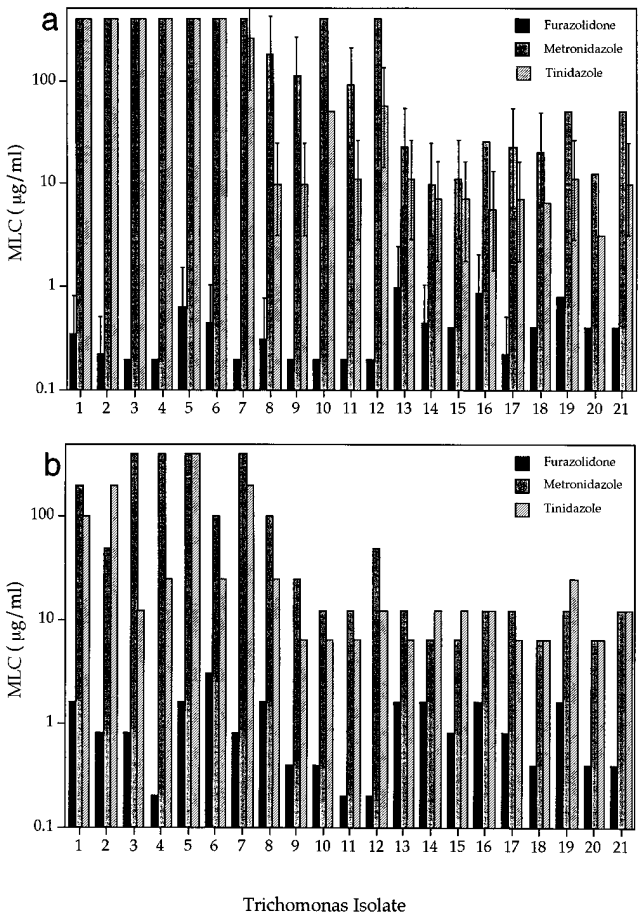

FIG. 1. Forty-eight-hour aerobic (a) and anaerobic (b) furazolidone, metronidazole, and tinidazole MLCs for well-characterized metronidazole-resistant organisms (isolates 1 to 7), recently isolated organisms from clinically metronidazole-resistant infections (isolates 9 to 12), and recently isolated organisms from clinically metronidazole-susceptible infections (isolates 13 to 21). Data represent mean MLCs and 95% confidence intervals for each isolate from two experiments performed in triplicate. MLC variability for a given isolate-drug combination was observed only under aerobic conditions. Drug concentrations are plotted on a log scale.

MLC time courses with the different isolates are shown in Fig. 2. All data are reported as means ± standard deviations to show the variability among isolates in the three categories: recently isolated clinically metronidazole-susceptible organisms (a and b), recently isolated clinically metronidazole-resistant isolates (c and d), and clinically metronidazole-resistant organisms maintained for several years after being isolated (e and f). For the clinically metronidazole-susceptible isolates, the aerobic MLCs of metronidazole did not drop below 400 μg/ml until after 6 h, while the aerobic MLCs of tinidazole and furazolidone declined within the first 3 h (Fig. 2a). In the anaerobic environment, MLCs of all drugs were lower and there was no lag in metronidazole effectivity (Fig. 2b). For the more recently isolated metronidazole-resistant isolates, higher concentrations of metronidazole were required to effect killing in the aerobic environment (>200 μg/ml) than were required to effect killing in the anaerobic environment. Less tinidazole than metronidazole was needed to kill these same isolates in both aerobic and anaerobic environments (Fig. 2c and d).

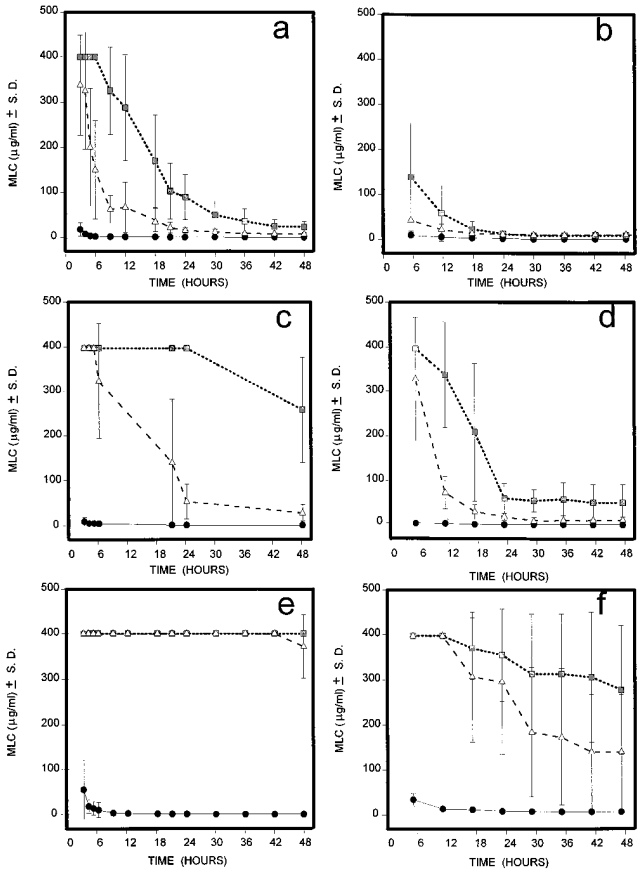

FIG. 2. Time course study of drug efficacy. (a and b) Recently isolated metronidazole-susceptible organisms ($n = 9$); (c and d) recently isolated metronidazole-resistant organisms ($n = 5$); (e and f) well-characterized metronidazole-resistant organisms ($n = 7$). Experiments for panels a, c, and e were performed under aerobic conditions, and those for panels b, d, and f were performed under anaerobic conditions. In the aerobic environment, 5-nitroimidazole (metronidazole and tinidazole) resistance is exacerbated (resulting in a higher MLC). Differences between aerobic and anaerobic MLCs were not seen with furazolidone. Graphs show the mean MLCs ± standard deviations (S. D.). ●, furazolidone; ■, metronidazole; △, tinidazole.

Higher concentrations of metronidazole and tinidazole (400 μg/ml or greater in the aerobic environment and 200 μg/ml or greater in the anaerobic environment) and longer exposure times (greater than 48 h in the aerobic environment and greater than 18 h in the anaerobic environment) were required to kill well-characterized metronidazole-resistant parasites (Fig. 2e and f). In contrast, furazolidone was effective against these same metronidazole-resistant organisms in both aerobic and anaerobic environments within 6 h of exposure (Fig. 2c to f). Thus, furazolidone was effective in killing all 21 isolates at low concentrations in a manner independent of isolate-specific metronidazole resistance, duration of in vitro cultivation, or the presence of oxygen.

At various points during the time course experiment, parasites were harvested, washed, and incubated in fresh media containing no drug. Removal of drug from the parasite culture did not reverse the action of any of the drugs, demonstrating that lack of motile organisms was a good indicator of trichomonad death. In contrast, upon examination at 48 h after placement of parasites into fresh media, parasite death was also observed in cultures which had been incubated with less drug than the MLC. If a drug was active against an isolate (i.e., this did not occur with metronidazole-resistant isolates incubated with metronidazole), concentrations killed isolates at an average of fourfold less drug (2 dilutions) than the MLC at that time point (data not shown). These results suggested that the microbicidal signal or "lethal hit" was delivered prior to the time at which the effect could be observed as an MLC.

DISCUSSION

In the trichomonad, metronidazole is reduced to its cytotoxic compound by a single electron transfer from ferredoxin to the nitro group of metronidazole (17, 24, 36, 40). The interaction between the reduced free radical drug and the organism's DNA results in strand breaks (49). Metronidazole-resistant organisms have reduced levels of intracellular ferrodoxin, ferredoxin mRNA, and ferrodoxin gene transcription compared with metronidazole-susceptible strains (40). Because ferredoxin levels and metronidazole resistance are inversely related, metronidazole resistance is considered relative and not absolute. As a result, clinically resistant trichomoniasis is often treated with an increased dosage of metronidazole for longer periods (6, 27–29, 38). However, many patients cannot tolerate these higher dosages of metronidazole and others are allergic to any concentration of the drug. This fact, along with an increase in the number of organisms resistant to metronidazole, has prompted the search for alternative antitrichomonal therapies.

A number of alternative therapies have been reported, with conflicting results concerning efficacy. These drugs include paromomycin sulfate (39, 47), clotrimazole (23, 25), povidone-iodine (50, 54), and acetarsol (12, 50). We report here two drugs, tinidazole and furazolidone, analyzed in vitro for their efficacy against metronidazole-resistant and -susceptible *T. vaginalis.*

Tinidazole, like metronidazole, is a 5-nitroimidazole. It is not available in the United States but is used in other countries to treat trichomoniasis. Many reports show excellent in vivo results with tinidazole therapy (13, 26, 51). However, a number of researchers have reported a similarity in mechanistic action between the nitroimidazoles by using in vitro assays (13, 24, 31, 51). Therefore, it is not surprising that various case reports concerning metronidazole-resistant trichomoniasis also show failure with tinidazole treatment, suggesting that the in vitro similarity is also seen in vivo (39, 53). There have also been a number of reports of patients allergic to metronidazole who are also allergic to tinidazole (18, 34). In the present study, tinidazole was effective against all of the metronidazole-susceptible organisms but effectively killed only 3 of the 12 metronidazole-resistant organisms. For the suspected metronidazole-resistant trichomonad samples that are sent to the CDC for metronidazole susceptibility testing, only about 30% of the metronidazole-resistant organisms assayed by the in vitro susceptibility assay appear to be susceptible to tinidazole (unpublished data). Although tinidazole does work in some cases of metronidazole-resistant trichomoniasis (26), the unavailability of the drug in some countries and the similarity of its mechanistic pathway to that of metronidazole makes it a poor alternative therapy for patients with metronidazole allergy or metronidazole-resistant trichomoniasis.

Furazolidone is a nitrofuran, presently marketed in the United States for use against enteric bacteria and *Giardia lamblia*. This drug is metabolized in part to 2-hydroxyethyl hydrazine, a potent monoamine oxidase inhibitor (48). This drug was sold in the early 1950s under the trade name Tricofuran by Eaton Laboratories. Treatment with Tricofuran entailed a combination of vaginal insufflation and a pessary, since

furazolidone is poorly absorbed from the digestive system (52). Clinical studies using this product reported excellent results. One study with 48 patients showed that within 72 h of the Tricofuran therapy, 45 patients (94%) had complete relief of symptoms and only 3 patients had viable parasites. At the end of 1 week, only one patient had motile trichomonads. In this study, there were three symptomatic recurrences within 1 to 3 months after the end of treatment, but these patients were cured with reinstitution of therapy (44). Another study used 25 patients with chronic vaginal discharge. Of these patients, 24 were completely cured, without recurrence. There was one failure due to patient noncompliance, but a repeat treatment was successful. A number of other studies had patients use only pessaries without the vaginal insufflation. In these studies, the cure rates were poorer and recurrences were noted (20, 43). One study was conducted with male trichomoniasis patients and used urethral irrigation with furazolidone for 10 days with a cure rate of 18 of 20 patients (1).

The results of the study presented here are similar to those of other studies of the in vitro efficacy of furazolidone. The initial evaluation of furazolidone's trichomonacidal activity in 1956 (14 isolates) and a more recent study which screened the susceptibility of 5 isolates to 50 antimicrobial agents showed that all isolates were susceptible to low concentrations of furazolidone (43, 45). This study extends these previous findings by demonstrating that furazolidone is effective against several clinically metronidazole-resistant isolates (only one metronidazole-resistant isolate had been tested previously) and that it can effectively kill these isolates in a very short time. The well-characterized drug-resistant organisms which had been in culture for years and the fresh susceptible and resistant isolates that had been in culture for less than 2 months all had similar low furazolidone MLCs. No resistance to furazolidone was noted in any of the organisms in this or previous studies. As furazolidone is already licensed for treatment of other infections in humans, it is unlikely to have severe side effects which would preclude its use to combat trichomoniasis. Therefore, furazolidone may be a useful alternative therapy for metronidazole-resistant *T. vaginalis* infections and for patients allergic or sensitive to metronidazole. Further studies testing its in vivo usefulness should be pursued.

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